Date: 4/12/01 Express Mail Label No. EL552577044US

Inventors: Seshi R. Sompuram and Halasya Ramanathan

Attorney's Docket No.: 1159.1008-005

QUALITY CONTROL FOR CYTOCHEMICAL ASSAYS

RELATED APPLICATIONS

This application is a continuation of Application No. 09/549,855, filed April 14, 2000, which is a continuation-in-part of Application No. 09/291,351, filed April 14, 1999. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant 1R43CA81950-01 from The National Institute of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

During the past decade, immunohistochemical (IHC) stains have become an integral part of the diagnostic process in surgical pathology. IHC stains are used with conventional histopathological stains, as adjunctive assays. They are critical in correctly diagnosing poorly differentiated malignancies, viral infections, and tumor prognoses, such as in the use of estrogen receptor (ER) analysis for breast carcinoma. Since IHC assays are relatively new in medical practice, simple methods for accurately and reliably

monitoring quality control has not yet been developed.

Traditional microscopic analysis of biopsy samples demonstrates overall cellular and tissue architecture. With the commonly used hematoxylin and eosin stain, for example, nuclei are colored purple (with hematoxylin) and the cytoplasm red/pink (with eosin). Often, the type of information available from such stains is insufficient for accurate diagnosis. IHC stains have the ability to extend the level of analysis on a biopsy sample, beyond cellular size and shape, to a molecular level. Tissue samples can be probed for the presence of specific proteins using monoclonal and polyclonal antibodies. The presence of such proteins can be indicative of the cellular lineage of a tumor, facilitating a diagnosis or prognosis with certain types of anti-tumor therapies. Alternatively, the presence of microbiologic agents, such as viruses or bacteria, can be detected using appropriate antibodies.

Quality control is an important aspect of any clinical assay. To assure that clinical test results are accurate, controls should be run with all *in vitro* diagnostic tests. Quality control in the clinical laboratory is mandated by the Clinical Laboratory Improvement Act of 1988 (CLIA '88). This act outlines the regulatory requirements that a clinical laboratory must meet in order to obtain accreditation; controls and proficiency testing comprise a significant portion of the act. Many tests in the hematology and chemistry sections of the laboratory include controls that are provided by the manufacturer. In contrast, most histology laboratories generate their own controls from excess tissue specimens. With laboratories generating their own control tissues, there is little inter-laboratory standardization. The need for better quality control of immunoreagents was recognized in the late 1980's and led to workshops convened by the Biological Stain Commission to address the issue.

The histology laboratory has lagged behind other sections of the clinical laboratory in the implementation of optimal controls. A negative control is easy to perform. It comprises a serial section of the same tissue with an irrelevant, isotype-

matched primary antibody. Standardized positive controls have been harder to achieve. Presently, each laboratory is left to fend for itself in creating, storing, and validation positive tissues controls.

Most histopatholoy laboratories use tissue samples previously documented to contain the particular antigen as positive controls. The laboratory documents, sections, and archives a bank of tissues that will serve as tissue controls for IHC. As the tissue controls are depleted, new tissues/tumor samples are procured to replace those expended. During each daily IHC assay run, each antibody is tested on a positive tissue control. In this manner, each antibody is validated at a specified dilution.

An improvement was described by Battifora (U.S. Patents 4,820,504 and 5,610,022), whereby multiple positive control tissue fragments, often of tumors, are embedded together in a single paraffin block. This "multi-tissue tumor block" simplifies the sectioning process of positive tissue controls. Rather than archiving and sectioning numerous blocks of tissue, the tissue controls are embedded together in a single paraffin block. Therefore, the group of archived tissues can be sectioned simultaneously, with a single stroke of a microtome blade.

A slightly simpler method of preparing multi-tumor tissue paraffin blocks was described by Furmanski et al. (U.S. Patent 4,914,022). The improvement involved embedding tissue cores in a paraffin block. The cores were cut from the tissue of origin with the use of an ordinary plastic drinking straw.

The use of multi-tumor tissue blocks as positive controls does not solve three important problems. One of the most important aspects that a positive control should address is the early detection of reagent failure. The ideal method of detecting early failure is to determine the level of sensitivity at the working concentration of antibody. Sensitivity is determined by titrating the antigen concentration until the antigen is no longer detected. In this manner, the assay can be stated as capable of detecting a certain amount of antigen, e.g., nanomoles or picomoles of antigen. The limit of sensitivity

should ideally be checked daily so that trends (towards increasing or decreasing sensitivity) can be detected. It is impossible to perform this type of analysis using tissue sections as controls since there is no practical method for quantitation and titration of antigen in a tissue section.

In addition, tissue sections as controls do not control for performance error. Cutting (with a microtome) and mounting tissue sections on glass slides is labor intensive. Therefore, the aforementioned types of tissue positive controls (such as multi-tumor tissue blocks) are usually tested once per assay run. Because of the associated labor costs, few laboratories place a positive tissue control on each microscope slide. Thus, if there is an error by placing an incorrect antibody (or no antibody) on the sample, it may be impossible to detect. Importantly, the control slide may be correctly treated (verifying the reagent quality) but the sample slide can still be incorrectly treated. The sample would therefore be interpreted as a negative result, although the cause of the negative result is an error in the assay procedure. The present system of positive tissue controls does not control for errors in procedure.

A third problem with tissue sections as positive IHC assay controls is that tumor tissues inherently have a varied, non-standardized amount of antigen. Therefore, tissues do not provide a ready means for calibrating the intensity of the immunologic reaction to an external reference standard. For certain IHC assays, the absence of external reference calibrators is a serious problem. Notably, IHC assays for estrogen receptor and progesterone receptor have become the gold standard for previously quantitative assays that were performed in a test tube. In the absence of such calibrators, staining is typically quantified as 0 - 4+ staining intensity, an arbitrary standard that depends upon the reagents, protocol, and time duration of colorimetric development. Because of significant inter-laboratory variability in IHC assay sensitivity, each hospital laboratory must develop its own threshold for determining a positive result. This feature leads to non-standard and sometimes incorrect results. These errors can have therapeutic impact

on patient care.

Therefore, a standardized, practical positive tissue control for clinical IHC assays should have the following characteristics to be clinically accepted and scientifically meaningful:

1. Antigen specific. A positive reaction should indicate the presence of only the antigen being assayed.

2. Available in virtually unlimited quantities, so that the positive control has constantly controlled characteristics with the passage of years.

3. Inexpensive. With cost pressures mounting on hospital laboratories, an expensive positive control will most likely not be broadly adopted into routine practice.

4. Stable over a prolonged period of time, ideally without the need for freezing.

5. Standardized, so that each laboratory will have the exact same positive control substrate.

Currently, there is not a quality control reagent or device available for cytochemical procedures that has all of the above characteristics.

SUMMARY OF THE INVENTION

The present invention relates to the development of accurate, reliable and easy-to-use quality control devices, and methods of using the devices, to maintain quality control of assays that measure analytes in cells or tissue sections, specifically immunohistochemical analyses of biological samples. As used herein, the term "biological sample" can be any cell-containing sample. For example, the sample can be tissue, blood, urine, cerebral spinal fluid (CSF), sputum, semen, cervicovaginal swab, or intestinal wash. For example, the analyte assay can be an immunocytochemical assay and the target molecule (analyte) to be detected is an antigen. As used herein, the term

"antigen" means a molecule detected by an antibody. The term "immunocytochemical" is used synonymously with "immunohistochemical", both referring to an antibody test for *in situ* identification of analytes in cells or in a tissue section.

The quality control device of the present invention is useful for monitoring quality control of assays that measure analytes in cells or tissue samples obtained from biological samples. Specifically, the present invention relates to a quality control device comprising one, or more, quality control moieties that are affixed, or attached, to a reagent surface of a matrix. The device comprises a matrix (a thin or single layer) having a front (also referred to herein as top) surface and a back (also referred to herein as bottom) surface. Typically, the quality control device is used in an assay that is performed on a flat, planar test platform, or surface (e.g., a glass microscope slide). The matrix can be adhered to the test platform by an adhesive on the bottom surface of the matrix. One example of a matrix is a membrane, such as nitrocellulose. Each moiety is confined to a discrete (and distinct) section, or "spot" on the matrix. Typically, the moieties are covalently attached to the matrix. The quality control moiety comprises one, or more, target molecules (e.g., the molecule of interest that is being detected in the biological sample) or a target molecule mimic (e.g., a synthetic molecule that mimics the target molecule). Typically the target molecule is a protein (e.g., a native protein, or an antibody such as a goat anti-mouse immunoglobulin antibody or normal mouse immunoglobulins) or polypeptide (e.g., synthetic protein, peptide, or antibody fragment) that is detectable by antibody binding. Other target molecules are proteins or polypeptides, carbohydrates, lipids, or combinations thereof that are detectable by histochemical stains. Representative histochemical stains include the periodic acid-schiff stain, mucicarmine stain or reticulin stain. Target molecules can also be nucleic acids (e.g., that are detectable by *in situ* hybridization techniques). For example, in this embodiment, the target is a nucleic acid detected by a nucleic acid probe complementary to the nucleotide sequence of the target molecule.

In one embodiment, the target molecule is a protein, polypeptide, or fragment thereof, that is detectable by a specific antibody (referred to herein as the primary antibody). The primary antibodies of the present invention can be either polyclonal or monoclonal, but are typically monoclonal . Such proteins, polypeptides or fragments (e.g., small peptides) are referred to herein as antigens. Antigens for use in the quality control device described herein can be purified antigens, recombinantly produced antigens or synthetic antigens. In order to simplify manufacture, it is desirable to avoid the need for purification of the many different antigens that are used in clinical diagnosis. Therefore, a preferred form of the antigen of the quality control device described herein is a synthetic, short peptide sequence that mimics the antigen to be detected and specifically binds to the primary antibody under substantially the same conditions as the antigen to be detected (also referred to herein as a target molecule mimic).

In this embodiment of the present invention, antigen is affixed to the quality control device, for example, by placing a small spot of soluble antigen (i.e., antigen in a suitable solution, such as a buffered saline solution) onto a spatially discrete region, or section, of the matrix. That is, the antigen is spotted onto the matrix in a discrete, limited area of the matrix, so that the antigen spots to not overlap or touch, nor do they overlap/touch the biological sample. The matrix can be any material suitable for permanently affixing the antigen, including glass. Preferably, a series of spots, each with soluble antigen in varying concentrations, is placed onto the matrix. In this way, the immunologic reaction on the matrix will cause some spots to be intensely colored and others not at all. For example, to determine the sensitivity of an immunocytochemical assay, the endpoint of detection is determined as the spot of lowest antigen concentration that still produces a 1+ intensity (on a 0 - 4+ scale). In this manner the sensitivity of the assay and initial stages of reagent failure can be determined. In addition, an irrelevant antigen can also applied to the matrix as a check

for reaction specificity. Such antigen spots can serve as quantitative and qualitative reference/standard controls for IHC assays.

Alternatively, different antigens can be affixed to the matrix in order to detect multiple distinct primary antibodies. In this alternative embodiment, the quality control invention would be more versatile, in that it would provide an antigen-specific binding site for multiple different primary antibodies.

In another embodiment of the present invention, the quality control moiety is an antibody that recognizes the primary antibody that binds to the target molecule. For example, the quality control moiety can be goat anti-mouse immunoglobulin (IgG) if the primary antibody is a mouse antibody, or rabbit anti-human antibody, if the primary antibody is a human antibody. In this embodiment, the quality control device monitors for the presence of primary antibody and whether immunocytochemical reagents used during the cytochemical assay (e.g., second antibody or enzyme) are applied in the correct sequential order.

In another embodiment of the present invention, the quality control moiety is normal mouse or rabbit immunoglobulins. These immunoglobulins can serve as a target for the secondary (detecting) reagent that is commonly used in immunohistochemical assays. Namely, the normal mouse or rabbit immunoglobulins would be recognized by anti-mouse or anti-rabbit IgG. The presence of normal mouse or rabbit immunoglobulins would therefore test the proper performance and sensitivity of the detection kit components in an immunohistochemical assay.

In one embodiment of the present invention, the quality control device is an adhesive device wherein the matrix of the device has a front and back surface. The front surface is referred to herein as the reagent surface and has one, or more, quality control moieties affixed to the surface. The back surface of the matrix is referred to herein as the adhesive surface, which permits the quality control device to be affixed to a test platform. The test platform can be a flat, optically transparent surface, for

example, a microscope slide. For simplicity, the quality control device is often referred to herein as a "strip" of matrix material, however, it is readily apparent that other shapes, or forms of the matrix can be used in the device of the present invention, and these alternative forms are also encompassed by the present invention. For example, the quality control device comprising an adhesive matrix strip can be peeled from a backing and applied to the end of a microscope slide. The biological sample (e.g., tissue section) to be tested is also affixed to the same microscope slide. Therefore, the same reagents, temperature, and humidity conditions that exist for the cytochemical reaction on the tissue section also apply to the synthetic antigen control moieties affixed to the device.

In another embodiment of the present invention, the antigens can be attached, or affixed, directly onto a planar test platform, e.g., a glass microscope slide. This alternative embodiment eliminates the need for a separate matrix that adheres to the test platform with an adhesive backing. One or more antigen spots can be directly attached onto an area, or section, of the glass slide, leaving an appropriately large area for placement of the biological sample. In one embodiment of the present invention, the antigen is covalently attached, or coupled, to the test platform. A variety of covalent coupling chemistries are described herein, and are also known to those of skill in the art. Typically, the coupling utilizes a silane coupling chemistry to glass. These coupling chemistries attach a protein, synthetic peptide, nucleic acid (suitably derivatized for attachment), carbohydrate, or other macromolecule to the glass. The use of this alternative embodiment, with macromolecules attached directly to glass, is used analogously to that with a matrix having an adhesive backing. The same molecules that are detected in the tissue (or cell) sample are also simultaneously detected in the spots containing the antigen (or antigen mimic, such as a synthetic peptide).

Methods of using the devices described herein are also encompassed by the present invention. For example, encompassed by the present invention are methods for determining the sensitivity of an assay for the detection of the presence or absence of

one, or more, target molecules in a biological sample. The method comprises simultaneously processing the biological sample and a quality control device described herein in the assay to detect the presence or absence of one, or more target molecules. As used herein the term "processing" means performing all the steps of an assay required to detect the presence, or absence, of the target molecule. For example, processing can mean performing the steps of an immunocytochemical assay to detect the presence of a target protein by contacting the protein with an antibody that specifically binds to the protein, under appropriate conditions wherein the antibody specifically binds to the target protein and detecting the antibody bound to the target (e.g., by detecting a colorimetric signal) wherein detection of the signal is indicative of the presence of the target protein, and lack of signal detection is indicative of the absence of the target protein. Such assay steps are well-known to those of skill in the art. For example, the device can comprise a matrix with quality control moieties with different concentrations of a target molecule or target molecule mimic, e.g., a synthetic antigen covalently attached to the matrix. The synthetic antigen mimics the antigenic site of the target molecule and thus is also recognized by same antibody that recognizes the target molecule. The processing results in the detection of target molecule in the sample and target molecule or target molecule mimic in the quality control moiety of the device using one of the detection methods described herein. The moiety that contains the lowest concentration of target molecule/target molecule mimic is then determined, wherein determination of the lowest concentration of detectable target molecule/target molecule mimic is indicative of the sensitivity of the immunocytochemical assay. Typically, the device is affixed to a flat, optically transparent surface, e.g., a microscope slide.

Also encompassed by the present invention is an immunocytochemical assay method for validating, or verifying, the proper performance of the assay, for example, an assay that detects the presence or absence of a target molecule in a biological sample.

The method comprises simultaneously processing the biological sample and a quality control device in the assay, wherein processing results in a detectable signal (e.g., a colorimetric signal) produced by the target molecule and by the quality control reagent moiety. As described above, the processing results in the detection of the signal, therefore detection of the target molecule in the biological sample and the target molecule/target molecule mimic in the quality control reagent moiety. As described above, the quality control moiety can be a synthetic antigen/target molecule mimic. The fact that the quality control reagent moiety develops a colorimetric signal provides independent validation that the assay on the biological sample was executed correctly. This is especially important in instances where the biological sample yields a negative result, i.e., no color development. The fact that the quality control strip yielded a positive reaction establishes that the result is a true negative and not due to errors in the procedure or problems with reagent quality. Thus validation is established because the biological sample and the quality control device are typically affixed to the same microscope slide. Therefore, both the tissue sample and the quality control device contacted the same series of reagents, for the same time and temperature.

Another method of the present invention encompasses an assay method (e.g., an immunocytochemical assay) for the determination of the concentration of a target molecule in a biological sample. The method comprises simultaneously processing the biological sample and a quality control device in the immunocytochemical assay as described above. The processing results in a detectable signal generated by the presence of the target molecule in the biological sample and target molecule/target molecule mimic in the quality control moiety. The detectable signal generated from the target molecule in the biological sample is compared with the detectable signal generated from the target molecule (or synthetic antigen mimic) in the quality control reagent moiety to determine the concentration of target molecule in the biological sample.

As a result of the invention described herein, quality control devices and

methods are now available to serve as a quality control tool to verify that a cytochemical, immunocytochemical, or in situ hybridization procedure was executed correctly. Use of the quality control device of the present invention verifies that each and every tissue sample received the correct reagents, in the proper sequence and timing, leading to proper staining. Moreover, it verifies the integrity of the reagents.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an en face view of a microscope slide with a patient label and patient tissue sample. Additionally, the drawing demonstrates where on the slide the adhesive antigen strip might be positioned.

FIG 2. is an en face view of the antigen strip showing the positions of the antigen spots.

FIG. 3 is an en face view of the antigen strip showing the degree of color development after a representative immunohistochemical assay.

FIG. 4 is a graphic representation of an experiment to determine linear assay range.

FIGS. 5A & 5B are graphic representations of the results of an experiment for the detection of an early reagent failure. Figure 5A describes a failure test for a primary antibody. Control strips with varying concentrations of ER peptide were subjected to IHC staining with various dilutions (1/50, 1/450, and 1/900) of anti-ER mouse monoclonal antibody 1D5.A 1/50 dilution is our standard optimal 1D5. Figure 5B describes a failure test for a secondary antibody. Control strips with varying concentrations of ER peptide ;were subjected to IHC staining with various dilutions (1/300, 1/1200, and 1/4800) of a biotinylated anti-mouse IgG secondary antibody.

FIG. 6 is a list of peptides, using the single letter codes for amino acids, that simulate the binding epitope of estrogen receptor antibody 1D5.

FIG. 7 is a chemical representation of the surface of a glass slide.

FIG. 8 is a chemical representation of the surface of a glass slide after derivatization with 3-isocyanato propyltriethoxysilane.

FIG. 9 is a chemical representation of the surface of a glass slide after derivatization with 3-aminopropyltriethoxysilane.

FIG. 10 is a chemical representation of the surface of a glass slide after initial derivatization as shown in FIG.9 and subsequent treatment of the glass slide with 1,4 phenyl diisocyanate.

FIG. 11 is a chemical representation of the surface of a glass slide after conjugation of a protein or DNA molecule (represented as a jagged line) via an amino group on the DNA or protein.

FIG. 12 is a chemical representation of the surface of a glass slide after conjugation of a carbohydrate or DNA molecule (represented as a jagged line) via a hydroxyl group (on the carbohydrate or DNA molecule).

FIG. 13 is a chemical representation of a mildly stable intermediate formed by the reaction of carbonyldiimidazole with an amine bound to the glass surface.

FIG. 14 is a chemical representation of a mildly stable intermediate formed by the reaction of methyl ethyl ketoneoxime (MEKO) carbonate with an amine bound to the glass surface.

DETAILED DESCRIPTION OF THE INVENTION

The present invention as herein described addresses the important quality control issues in cytochemical assays, and in particular in immunohistochemical (IHC) assays. Most histopathology laboratories use tissue samples previously documented to contain particular antigens of interest (target antigen) as positive controls.

One of the most important aspects that a positive control should address is the early detection of reagent failure. The ideal method of detecting early failure is to determine the level of sensitivity of the working concentration of antibody. Sensitivity is

determined by titrating the antigen concentration until the antigen is no longer detected. In this manner, the sensitivity of the assay can be stated as the limit of the assay to detect a certain amount of antigen, e.g., nanomoles or picomoles of antigen.

The limit of sensitivity should ideally be checked daily. In this manner, long-term trends (towards decreasing or increasing sensitivity) can be detected. It is impossible to perform this type of analysis using tissue sections, since there is no practical method for quantitation and titration of antigen in a tissue section.

Immunohistochemical assays are inherently more prone to operator performance error than conventional immunoassays. This creates an additional problem with the existing systems of quality control. Namely, even if the control tissue slide appears satisfactory, how is a performance error detected on another (sample) slide?

There are several reasons that IHC assays are predisposed to operator error. First, the fact that the IHC reaction is performed on a microscope slide - - a flat planar surface -- allows for the possibility that the reagent may fall off the edge of the slide or evaporate, which would result in unsatisfactory staining due to tissue drying. Conventional immunoassays, performed in test tubes, cuvettes, microwells, or the like, have supporting walls for the reagent. This physical constraint for the reagent prevents reagent spillage.

Additionally, many clinical laboratories still perform IHC staining manually. This inherently creates the possibility that the technologist can place an incorrect reagent, or no reagent, during one of the many steps in the procedure.

Even with an automated platform, which are recently gaining market acceptance, machine malfunction is reported on an anecdotal level. Each of the machine platforms assumes that an electromechanical action translates into a dispensed volume of reagent. This is not always the case. For example, an automatic reagent dispenser can have several potential failure modes. These include insufficient priming of the dispenser, a leaking dispenser due to a faulty seal, and excessive friction (creating a drag force) by

the plunger in the reagent reservoir. Standard XYZ axis pipettors depend upon the technician placing a sufficient amount of antibody into a vial at the beginning of each run.

The final step in IHC staining is colorimetric development, catalyzed by an enzyme such as peroxidase or alkaline phosphatase. A colorless and soluble substrate is converted into a colored and insoluble precipitate. The timing of the reaction is important. Too short a development yields a poor signal while too long a development time can increase non-specific background staining. Consequently, this step is performed under the watchful eye of the technologist, who examines the tissue sections for color development under the microscope. When the color is optimally developed, with low background, the technologist stops the reaction by immersing the slide in buffer. In practice, this step is labor-intensive, since each slide is examined individually.

The use of a quality control device with every assay, or on every microscope slide, would solve the assay performance problem. Described herein is a quality control device comprising multiple quality control reagent moieties affixed to a matrix or membrane. The quality control device can be affixed, or adhered, to a test platform, such as a microscope slide, using an adhesive. These reagent moieties comprise the target molecule (e.g., the antigen, antibody or nucleic acid to be detected in the assay), or a target molecule mimic, defined herein as a molecule that mimics the characteristics, or properties (e.g., physical structure or antigenic properties) of the target molecule sufficiently so that the mimic molecule reacts substantially the same as the target molecule in the cytochemical assay. In one embodiment of the present invention, the target molecule mimic is a short synthetic peptide that mimics the antigen site of the target molecule and therefore, binds to antibody specific for the target molecule (also referred to herein as a synthetic antigen or synthetic control).

In one embodiment of the present invention, the quality control device comprises a membrane strip, approximately 0.8 x 0.2 inches, with an adhesive backing

that can be applied to a microscope slide. Figure 1 demonstrates a recommended position on the microscope slide (1) for such a control strip (2). At one end, a label (3) with patient information is typically applied. The information on the label typically includes the type of stain, surgical accession number, patient name, and occasionally a bar code. The tissue section (5) is typically placed in the middle of the slide. Immunohistochemical reactions are performed by the sequential incubation and removal of a series of reagents to and from the tissue section (5). By placing the control strip (2) near the tissue section (5), the strip will also contact the same reagents, as they are applied and removed.

Figure 2 demonstrates the placement of antigen on a representative antigen control strip. A series of spots of antigen (7 - 12) are evenly spaced across the length of the strip. Each spot comprises a macromolecule, preferably a peptide, immobilized onto the membrane matrix. In a preferred embodiment, spots 8 - 12 comprise the same peptide at different molar concentrations immobilized onto the membrane matrix. Therefore, if an immunohistochemical assay is known to have a threshold of sensitivity of 10 nanograms per mm^3 of antigen X, then spot 10 would ideally have such a concentration. Spots 11 and 12 would have progressively increasing concentrations (e.g., 30 ng/mm^3 and 90 ng/mm^3, respectively). Spots 9 and 8 would have progressively decreasing concentrations (3 and 1 ng/mm3). Spot 7 is designed to test specificity rather than sensitivity. Therefore, spot 7 would comprise an irrelevant antigen (antigen Y) immobilized onto the membrane matrix. Ideally, the concentration of antigen Y in spot 7 will be higher than the threshold concentration for detecting antigen X (10 ng/mm^3).

Figure 3 demonstrates the expected colorimetric result of the antigen spots after an immunohistochemical assay. The assay sensitivity and peptide concentrations are as stated in the above paragraph. Spots 10 - 12 are increasingly dark colored, owing to the increasing concentrations of peptide antigen. Spots 8 and 9 are colorless, since their

concentration is stated to be below the level of detection. Spot 7 is colorless, since the antibody to antigen X does not bind to antigen Y (comprising spot 7).

MATRIX

The quality control reagent moieties are affixed to a matrix or membrane as described herein. The matrix can be manufactured of materials capable of binding macromolecules. Suitable matrix materials include glass, nylon, nitrocellulose, and polyvinylidenedifluoride (PVDF). Nitrocellulose is commonly used for Western (protein), Northern (RNA) and Southern (DNA) blots, and is capable of binding macromolecules through non-covalent bonds. PVDF and nylon have greater tensile strength than nitrocellulose and can be derivatized to allow covalent coupling of macromolecules. Such matrices are commercially available and sold as Immobilon™ AV (Millipore Corporation, Bedford, MA) or Immunodyne membrane (Pall Corporation, East Hills, New York). Both matrices are derivatized by the manufacturer so that there is an abundance of free carboxyl groups on their surface. This allows for the coupling of macromolecules to the carboxyl groups through an amine or a sulfhydryl group.

Macromolecules are coupled to the matrix in the present invention by placing (e.g., spotting) a small aliquot (typically less than one microliter) of the macromolecule-containing solution on top of the activated matrix. The macromolecule, such as the antigen or peptide, is dissolved in a coupling buffer. The coupling buffer is preferably at a pH that maximizes the reaction of the macromolecule to the derivatized (or "activated") matrix. A preferred buffer is 0.5M Potassium phosphate pH 7.4. The reaction is performed at room temperature. Five minutes to an hour is a preferred period of time for coupling, for convenience of manufacture and typically results in approximately 80% coupling efficiency. The matrix is then rinsed with buffer to remove any unbound macromolecules.

The matrix is spotted with a series of dilutions of the relevant target molecule or target molecule mimic (e.g., synthetic peptide antigen). Different concentrations of the antigen (peptide), will be useful in quickly estimating the extent of colorimetric development without the need for microscopic examination. For example, the synthetic peptide control concentrations can be appropriately calibrated so that a 3+ control reaction will yield a strong tissue signal with minimal background. The amount of antigen blotted onto the matrix would need to approximate the amount of antigen present in the tissue section. This can be empirically determined by coupling sufficient antigen to the matrix so that the color development on the matrix approximately parallels the intensity of the color development in the tissue section itself.

Typically, the first spot is the antigen at the highest concentration, and is expected to yield a strong positive reaction (e.g., detectable signal) after IHC staining. Subsequent serial dilutions vary from two to twenty-fold in concentration. It is desirable to have a clearly defined limit of sensitivity. Specifically, the user of this control strip should be able to view the immunologic reactions that occur on the strip, and unequivocally identify an endpoint of sensitivity. The colorimetric IHC reaction product is strong above the limit of sensitivity and significantly weaker or absent below it. Therefore, the antigen dilutions are sufficiently different from each other so that there is a sharp falloff in immunoreactivity, creating a clearly-defined limit of sensitivity. A preferred embodiment of the invention is to spot the series of dots with antigen at approximately 3 - 6 fold serial antigen dilutions. An irrelevant antigen of similar size and charge characteristics is also spotted at the end of the strip. The irrelevant antigen provides a control for specificity of the immunologic reaction.

Additionally, a semi-quantitative determination of the concentration of a target molecule in a biological sample can be ascertained by comparing the detectable signal (e.g., color) generated from the antigen present in the biological sample and the detectable signal generated from the antigen in the quality control reagent moiety. The

closest match of detectable signals between the sample and the control is indicative of the approximate relative concentration of the antigen in the sample.

The relevant antigens are spotted on the matrix surface using micropipettes or precision syringes. This can be accomplished in an automated fashion using an array of micropipettes that repetitively apply a defined number of microliters to defined positions on the quality control strips (matrix).

An alternative embodiment of the invention is to place multiple, distinct antigens onto the matrix. In this alternative, a single quality control device might have all of the relevant antigens for a particular disease state. For example, one device might be for a lymphoma IHC panel and contain antigens for many of the relevant antigens for a lymphoma diagnostic work up. Another device can be for a soft tissue tumor IHC panel, another for an undifferentiated "round, blue-cell" tumor work up, etc. In this fashion, the synthetic control devices are more broadly applicable to many different monoclonal primary antibodies.

After the coupling procedure, the "activated" (derivatized) matrix still contains a high density of available active surface groups (e.g, carboxyl groups) for coupling to macromolecules. According to one manufacturer (Millipore Corporation, Bedford, MA), a carboxyl group is present every seven Angstroms on the matrix. Therefore, only a small proportion of the active carboxyl sites on the matrix are typically occupied by a macromolecule after coupling. The remaining carboxyl groups must be "quenched" (or "capped"), blocking any future potential reactivity. The matrix is quenched with any of a variety of small molecules that have free amino groups. Suitable quenching agents include ethanolamine, ethylenediamine, dithiothreitol, aminoethanediol, and aminopropanesulfonate. Alternatively, proteins such as gelatin or casein or amino acids such as glycine, can quench the matrix. A recommended quenching procedure is to cover the membrane with 10% v/v monoethanolamine in 1.0 M Sodium Bicarbonate buffer, pH 9.5. The matrix is incubated with the quenching solution for few minutes to

an hour at room temperature with constant agitation. The quenching solution is then aspirated or drained and the matrix rinsed with 0.01 M Sodium Phosphate, 0.14 M NaCl, pH 7.4, 0.1% v/v Tween 20 (PBS-Tween). Each wash is for five to 30 minutes with constant agitation. The wash solution is then aspirated or drained, and the matrix is allowed to dry.

ANTIGEN

Two forms of antigen can be used as a synthetic positive tissue control. First, the whole antigen can be coupled to the membrane. A list of some of the most commonly used antigens for clinical IHC diagnosis is shown in Table 1. Some of these antigens are available commercially. Examples include human immunoglobulins, prostate specific antigen, S-100 antigen, alpha-fetoprotein, and carcinoembryonic antigen, available from Fitzgerald Industries International, Inc., Concord, MA. Other antigens can be isolated by solubilization of cultured cells or tissue homogenates and purification by affinity chromatography. Affinity purification methods are well known to those skilled in the art.

Antigens can also be obtained by recombinant DNA methods. cDNA constructs encoding the desired antigen can be transfected into suitable host cells, expressed and purified using techniques well-known to those of skill in the art.

Alternatively, a short peptide can serve as the antigen. Suitable peptides are typically approximately ten amino acids in length, but can be longer or shorter in length and still specifically bind antibody. Antibodies only bind to a small region of an antigen, typically comprising a few amino acids. Antibody epitopes of protein antigens can be broadly classified as sequential ("linear") or conformational ("discontinuous"). This classification is based on whether or not the amino acids that interact with the antibody are positioned immediately adjacent to each other in the linear amino acid sequence of the native protein. The surface of the antigen that interacts with the

antibody can thus either consist of amino acids that are adjacent to each other or of amino acids that are separated in the primary sequence but brought together as a result of the natural folding (conformation) of the protein to its native shape. Epitopes consisting of residues close together in the primary sequence are called linear, continuous, or sequential epitopes, whereas epitopes consisting of residues separated in the primary sequence are called discontinuous or conformational epitopes.

Short peptides can substitute for antigens when monoclonal antibodies are used. Monoclonal antibodies have a single, defined binding epitope. Different monoclonal antibodies often bind to distinct epitopes of an antigen. Therefore, when short peptides are used as antigens in the synthetic positive control device, they are specific to the monoclonal antibody that is being used. Each monoclonal antibody requires a distinct peptide sequence as a surrogate antigen. Therefore, the synthetic positive controls, using short peptides as antigen, are both antigen-specific as well as antibody-specific. Several of the relevant peptides for antigens in Table 1 have been identified for specific monoclonal antibodies. For example, the 20 amino acid tandem repeat in the extracellular domain of polymorphic epithelial mucin (cancer-associated antigen CA15-3) is shown below, each letter representing an amino acid.

TABLE 1. COMMON ANTIGEN TARGETS FOR CLINICAL IHC ASSAYS

PROGNOSTIC MARKERS

Estrogen receptor

Progesterone receptor

p53 protein

Ki-67 protein

Proliferating cell nuclear antigen (PCNA)

HEMATOLOGIC MARKERS

CD3

CD15

CD20

CD30

CD34

CD45

CD45RO

CD99

Kappa light chain

Lambda light chain

Factor VIII

EPITHELIAL DIFFERENTIATION MARKERS

Prostate specific antigen (PSA)

Prostate specific alkaline phosphatase (PSAP)

Cytokeratin

Epithelial membrane antigen (EMA)

Carcinoembryonic antigen (CEA)

Polymorphic epithelial mucin

Mesenchymal differentiation markers

Desmin

Vimentin

Actin

Collagen type IV

MELANOCYTIC MARKERS

S-100

HMB45

MISCELLANEOUS

Neuron-specific enolase

Glial fibrillary acidic protein

Chromogranin

Synaptophysin

The binding sites for the DF3 (DAKO Corporation, Carpinteria, CA) and B27.29 monoclonal antibodies are shown above and below the sequence (SEQ ID NO:1). The relevant region is indicated by the dotted lines. (Reference: Bon GG, von Mensdorff-Pouilly S, Kenemans P, van Kamp GJ, et.al. Clinical and technical evaluation of ACS BR serum assay of MUCI gene-derived glycoprotein in breast cancer, and comparison with CA15-3 assays. Clin. Chem. 1997 43:585-593).

--------DF3 ------

-P A H G V T S A P D T R P A P G S T A P-

--------B27.29-------

Two general methods for identifying relevant peptides are known to those skilled in the art. One of these methods is the use of fragments of the protein antigen that are identical to one or more portions of the linear sequence. Sequence mapping technologies such as Multipin Peptide Synthesis Technology (Chiron Mimotopes, Victoria, Australia) enable linear sequence mapping by creating overlapping series of peptides on a 96 pin plastic structure. In this method, the primary sequence of the protein must be known. Using automated peptide synthesis equipment, overlapping

fragments of the protein are synthesized on the various pins. For example, pin #1 might have the first ten amino acids. Pin #2 would have amino acids 2-11, pin #3 amino acids 3-12, etc. If the monoclonal antibody recognizes a linear epitope, then it will probably recognize and bind to one or more of the protein fragments on the pins.

From general experience with peptide mapping, only approximately 5-10% of monoclonal antibodies bind to linear epitopes. The remaining epitopes are conformational (discontinuous). Therefore, an alternative method of identifying antibody-binding peptides is required. To identify discontinuous epitopes, the preferred method of peptide identification is known as phage display. Phage display, the display of genetically encoded diversity on the surface of M13 filamentous bacteriophage, allows the production and screening of tens of millions of proteins and peptides in a few weeks (Ladner, R. and S. Guterman, in W090/02809 (1990), the teachings of which are herein incorporated in their entirety by reference.; Ladner, R., et al., Directed Evolution of Novel Binding Proteins, (1993) U.S.; Wells, J. and H. Lowman, Curr. Op. Struct. Biol., (1992) 3(4): p. 355-362) (McLafferty, M., et al., Gene, (1993) 128:p.29-36; Clackson, T. and J. Wells, TIBTECH, (1994) 12: p. 173-184), the teachings of which are herein incorporated in their entirety by reference. The organisms containing those peptides that have the desired binding characteristics can be replicated, allowing repeated screening with increased stringency and amplification of the ligands. After a few rounds of screening and amplification, the peptides that remain are the higher affinity binders to the target molecule. Phage display is recognized as an efficient method of producing proteins and peptides that bind to targets of interest. (Ladner, R. and S. Guterman, in International patent application W090/02809 (1990); Ladner, R., et al., Directed Evolution of Novel Binding Proteins, (1993) U.S.; Roberts, B., et al., Proc. Natl. Acad. Sci. USA, (1992) 89: p. 2429-2433; Markland, W., A. Ley, and R. Ladner, Biochemistry, (1996) 35: p. 8058-67; Markland, W., et al., Biochemistry, (1996) 35: p. 8045-57; Ley, A., W. Markland, and R. Ladner, Mol Divers, (1996) 2: p. 119-24, the

teachings of which are herein incorporated in their entirety by reference.

ADHESIVE BACKING

One embodiment for the synthetic antigen control strips is that they peel off from an 8.5 x 11 inch sheet. Each sheet would contain a plurality of control strips. The user simply peels off the appropriate antigen control from the sheet and applies it to the end of the slide, Therefore, the patient's name and identifying information would be at one end of the slide, and the antigen control at the other. Each strip would be spotted with several different concentrations of antigen.

Commercially available nitrocellulose, nylon or PVDF membranes can be used to create adhesive strips using techniques established in the printing industry. Specifically, a pressure-sensitive adhesive is applied to the back of the membrane. A suitable adhesive is FASSON S727 acrylic adhesive, because it is waterproof and permanent. Backing paper is then applied to the adhesive-coated nylon membrane. A suitable backing paper is 50 pound Kraft paper with a silicone liner. The purpose of the backing and liner is to provide a surface from which the membrane strips can be peeled. When the membrane strips are peeled away from the backing, the adhesive largely remains with the membrane. The application of adhesive and backing paper is typically performed by machinery that starts with two rolls (one roll of nylon membrane and backing paper, each) and ends with one roll of the nylon-adhesive-backing paper sandwich.

An alternative method of fabricating a membrane with an adhesive backing is to apply a double-faced tape to the back of the membrane. The double-faced tape comprises a film with adhesive coatings on both sides. A release liner covers the adhesive on one side and the other adhesive side is applied to the back of the membrane. Suitable double-sided tape can be obtained commercially, such as from Adhesives Research, Inc., Glen Rock, PA. Suitable tapes generally use acrylic adhesives and

polyester films. Acrylic adhesives are preferred because they exhibit a high degree of solvent resistance and are biologically inert. Examples of suitable tapes include their model Arcare® 7737, 8570, 7840, and 7841. The membrane and double-sided tape are applied to each other by machinery well known in the printing and labels industry, where each starts out as a separate roll. Each spool feeds its film or membrane into a roller that compresses the tape against the membrane. The hybrid sandwich of membrane and tape is then taken up on a third spool.

By placing the control strip against a liquid impermeable surface (an adhesive backing on top of a glass slide), vertical (downward) flow of reagent from the reagent surface of the strip inwards is prevented. The adhesives often have a hydrophobic character preventing water penetration. If double-sided tape is used, the tape described herein is also water impermeable. The glass slides are also impermeable to water or other liquids. This feature is in contradistinction to many other blotting techniques where vertical flow through the membrane into adsorbent paper towels is helpful. By preventing vertical flow of reagent, the strips exhibit an increased sensitivity of antigen detection, reasonably attributed to a longer incubation of the reagent with the quality control moieties immobilized to the reagent surface of the matrix.

USE OF THE QUALITY CONTROL DEVICE

In practice, the technologist typically mounts a tissue section, cytospin, or cellular smear on a glass microscope slide so that the stained specimen can be visualized with a microscope. These techniques are well-known to those of skill in the art. The quality control strip is applied to the same glass slide, near or adjacent to the specimen. In this manner, the strip receives the same series of reagents, for the same incubation times, temperatures, and washing conditions as the biologic specimen. For immunohistochemical assays, a series of antibodies are applied to a biologic sample, such as a tumor section. The first antibody is for the purpose of conveying the assay

specificity. A list of many commonly used antibody targets is listed in Table 1. Typically, the antibodies will be monoclonal, of murine origin. The primary antibody is commonly incubated with the tissue section for a period of about 15-60 minutes.

The remaining reagents in the assay are considered detection reagents. They demonstrate where in the biologic specimen the first (primary) antibody bound, by causing that site to become colored. After the primary antibody incubation, the slide is rinsed with a buffer, commonly phosphate buffered saline, in order to remove any unbound antibody molecules from the surface of the slide. Then, a secondary antibody is applied. A common secondary antibody is a polyclonal anti-murine IgG or IgM recognizing unique epitopes present on murine immunoglobulins. This secondary antibody is commonly coupled (covalently) with biotin. Consequently, wherever the primary antibody bound to the biologic specimen, biotin molecules are now found. After the secondary antibody incubation, excess unbound antibody reagent is rinsed off with a buffer. The third step in the reaction sequence provides for coupling of an enzyme, typically peroxidase, to the site where the primary antibody bound. This is commonly performed by incubating the slide with avidin (or streptavidin) covalently coupled to peroxidase. Avidin (and streptavidin) has a high binding affinity to biotin. Therefore, peroxidase will be immobilized at sites of biotin. After completion of this third step in the reaction, the excess unbound enzyme - avidin reagent is rinsed off with buffer. The final step is the addition of an enzyme substrate. Substrates are chosen so that they are soluble and relatively colorless. However, after action of the enzyme upon the substrate, they become colored and insoluble. Therefore, the substrate precipitates wherever the enzyme is found. A commonly used peroxidase substrate is 3,3-diaminobenzidine.

Numerous other variations of the immunohistochemistry procedure exist and are described in the relevant literature. This quality control strip is designed so that it will serve as a positive control regardless of the detection system used. Whatever series of

reactions that occur on the biologic sample will also occur on the quality control strip. The use of more sensitive detection systems will correspondingly be reflected in the increased color intensity of both the biologic sample and the quality control strip. Moreover, a more sensitive detection system will detect the lower concentration spots of antigen. The opposite will be true of less sensitive detection methods. In this manner, the quality control strips can verify and quantify the performance characteristics of immunohistochemical assays.

Although immunohistochemical assays have been specifically referenced in the examples, the same quality control method can also be applied to other types of assays performed on microscope slides. For example, nucleic acid targets can be immobilized onto the matrix and serve as positive controls for assays such as *in situ* hybridization. In this configuration, probes that detect specific nucleic acid sequences will also hybridize with complementary sequences on the quality control strip. Such assays are well known to practitioners in the field and published in scientific literature. Commonly performed *in situ* hybridization assays test for the presence of viruses. As a control, dsDNA containing the desired double-stranded sequence is covalently coupled to the matrix. A convenient method for coupling dsDNA is to derivatize it at one end with a free amino group. The free amino group can then be coupled to the carboxy-derivatized matrix in the same fashion as already described.

This quality control method can also be applied to the field of histochemical stains. Classes of compounds that are detected by histochemical methods can be immobilized onto the matrix. Examples include carbohydrates for the Periodic acid-Schiff (PAS) stain, mucins for the mucicarmine stain, extracellular matrix components for the reticulin stain, etc. Another application of this quality control method can be in the diagnosis of microbes or parasites. Whole microbes, or purified or recombinant microbial or parasite specific antigens or antigen-mimics can be similarly used as quality control antigen strip on the slide. Such microbial controls can serve as controls

for cytochemical stains used in microbial detection, such as in the Gram, Steiner, or methenamine silver stains. Alternatively, macromolecules from these microorganisms (or antigen mimics) can serve as controls in immunohistochemical or nucleic acid diagnostic assays for these microbes.

With respect to the five criteria previously mentioned, the quality control devices described herein are antigen-specific, easily manufactured in large quantities, inexpensive, stable and standardized. The antigenic specificity is conferred by virtue of the antigen that is immobilized onto the antigen strip or glass slide. Short peptides (e.g., approximately 5-50 amino acids long, and more typically approximately 10-20 amino acids long) are easily manufactured at low cost. Moreover, they tend to be quite stable, especially if stored at 4° C. By applying a calibrated amount of antigen to the strip, under reproducible coupling conditions, the amount of antigen bound can be standardized.

The synthetic controls described herein provide an absolute quantitative standard for immunohistochemical reactions. The antigen strips or glass slides are manufactured so that a series of known concentrations of peptide are deposited, as previously described. This provides laboratories the means to verify that they each have comparable assay sensitivity. For example, estrogen receptor analysis is commonly performed by visually quantifying (under microscopic examination) the percentage of tumor cells that stain positively using an antibody to estrogen receptor. However, a significantly more sensitive assay will be expected to detect more positive cells than a less sensitive assay. Therefore, assay sensitivity standardization is important for laboratories to meaningfully communicate their results. Since the synthetic antigen control strips are manufactured in a standardized and reproducible fashion, each laboratory has a reproducible assay standard.

In summary, the quality control devices described herein can serve three functions:

1. VALIDATION OF PROPER ASSAY PERFORMANCE.

The devices contain analytes that are identical to or mimic the proteins, peptides, nucleic acids, carbohydrates, lipids, etc. found in the cell or tissue sample that is being analyzed. These analytes produce a substantially similar colorimetric signal as the signal produced by the staining reaction on the cells or tissue section. Therefore, if the quality control device yields a positive signal, it serves as an indicator that the staining procedure was performed correctly. This is particularly useful in instances where the analytes being tested for in the cell or tissue sample are not present, e.g,. it is useful in assays that produce a negative tissue reaction. A positive reaction on the quality control device establishes that the negative result in the tissue sample is a true negative rather than being due to errors in the staining procedure.

2. DETERMINATION OF ASSAY SENSITIVITY.

The devices that contain varying quantities of the analytes provide a method for establishing the endpoint of sensitivity of the assay, e.g., the lowest detectable concentration of analyte on the control device that is detectable represents the threshold of sensitivity.

3. QUANTITATION OF ANALYTES IN THE CELL OR TISSUE SAMPLE.

The devices that contain varying quantities of the analytes can provide a concentration reference standard that can be used for analyte quantification. Specifically, the colorimetric signals can be expected to vary according to the analyte concentration. Low analyte concentrations will yield weak colorimetric signals; high concentrations will yield strong colorimetric signals. The intensity of the signal can either be estimated visually or directly quantified using computer-assisted image analysis techniques. With either method of

quantification, the signal intensity on the quality control device can be correlated with the signal intensity found in the tissue section. In this manner, the approximate amount of analyte in the cell or tissue section can be estimated. If quantitative signal measurement methods are used (image cytometry), then a calibration curve can be established every time the assay is run using the quality control device, e.g., the signal intensity is plotted (y axis) against the analyte concentration (x axis). The signal intensity in the cells or tissue section can then be interpolated on this graph to deduce the analyte concentration.

The following examples will further illustrate the claimed invention.

Example 1: Select phage that mimic target epitopes from monoclonal antibodies commonly used in clinical immunohistochemical laboratories.

For the device and methods describe herein, it is desirable to identify peptides that will mimic the antigen-specific interaction of antibody with the native antigen. These peptides will be suitable for use on either the quality control adhesive strips or glass slides described herein. Suitable peptide sequences identified as described herein are more amenable to consistent manufacture than the native protein itself. The monoclonal antibody clones suitable for use as described herein are those that recognize the antigen even after the tissue has been fixed with formalin and embedded in paraffin wax. These monoclonal antibody clones represent some of the most commonly used primary antibodies in the clinical IHC laboratory. The cyclic peptide libraries comprise a random assortment of peptides that have two cysteine residues that are linked by a disulfide bond. This intramolecular bond imparts the advantage of a better-defined and more stable three-dimensional structure. The DNA sequence coding for the random peptides are inserted into the Gene 3 phage protein, available from Dyax Corp., Cambridge, MA.

(a) Screening of peptide and protein display libraries.

The following procedure is used to select phage from libraries that bind specifically to antibody combining sites. Selection of phage is effected through binding of phage to target mouse monoclonal antibody that is captured onto human anti-mouse IgG-conjugated paramagnetic beads (CELLectin Pan Mouse IgG Dynabeads, available from Dynal Inc., Lake Success, N.Y.)

The human anti-mouse IgG-conjugated magnetic beads were capable of capturing approximately 0.5 to 2 μg of mouse IgG from a hybridoma cell culture supernatant. Captured antibody can be eluted with 0.1 M HCl. Peptides displayed on phage particles that mimic the epitope of specific target molecule recognized by a specific monoclonal antibody are screened. In essence the peptide binds to the antibody combining site in a similar fashion as would the target antigen.

At the start of the panning procedure, non-specifically binding phage are initially removed by five rounds of negative selection against magnetic beads coated with normal mouse IgG. This step removes phage that bind to framework determinants of murine IgG. Approximately 100 microliters of CELLectin Pan Mouse IgG Dynabeads coated with normal mouse IgG are resuspended in 500 μl of a PBS-Tween 20 (0.05%) buffer with 10^{11} phage particles that comprise the starting phage library. The beads and phage particles are incubated at 25°C for 1 hour, with gentle tumbling.

After the initial rounds of negative selection, the positive selection of desired phage particles is then performed. The target mouse monoclonal antibody is captured onto Pan Mouse IgG Dynabeads (human anti-mouse IgG-conjugated magnetic beads). The negatively depleted phage particles are then incubated with the antibody-coated beads. Bound phage are eluted by acidification (pH 2, by addition of 150 μl of 50 mM glycine-HCl) of the bead pellet. The beads are incubated for 15 minutes at 25°C in the pH 2 buffer. The acid-eluted phage are harvested from the supernatant and the pH neutralized by addition of 150 μl of 200 mM $NaP0_4$ buffer, pH 7.5. The positively-

selected phage are then grown for further rounds of panning.

(b) Enrichment for clones that contain high affinity binders to target antibody.

The phage libraries contain between 10^8-10^9 distinct phage particles. At the outset, there are approximately 100-1000 copies of each phage particle. Therefore, there are a total of approximately 10^{10}-10^{11} phage particles in the first screen. The goal of enrichment is to select only those phage particles that bind specifically and with high affinity to the target antibody. Typically, between 10^5-10^6 binding phage are recovered during the first round of screening. Many of the phage often represent redundant particles.

This pool of phage is then amplified and further screened. The enriched phage library is much less complex than at the outset, since this second round is pre-selected for binding phage. Therefore, the fraction of binding phage is usually to be greater than in the first round. Since this second round of amplification starts with the same number of total phage (after amplification of the first round of selected phage), up to 10^7-10^8 phage particles are expected to be obtained after the second round. Each round of screening should yield a greater number of binding phage, indicating enrichment. When the number of phage recovered has leveled off, this is an indicator that no further enrichment is occurring.

The eluted phage are used to inoculate 5 ml of a 1: 100 dilution of an overnight culture of TG1 or XL-1 Blue *E.coli* in 2X YT media, which are then grown for no more than 8 hours at 37 °C, shaking at 220 rpm. The cells are pelleted by centrifugation at 5000 rpm in a Sorvall Superspeed T21 tabletop centrifuge, and the phage in the supernatant are recovered by precipitation with polyethylene glycol. The selection is then repeated. Four rounds of selection are usually sufficient to enrich the pool for phage that contain high affinity binders to the antibody (though some phage screens

gave high binders after only two rounds of selection). For increased selection pressure, the stringency conditions for binding phage can be increased (e.g., by increasing the salt concentration or adding non-ionic detergent or gradually decreasing the concentration of target antibody molecule).

From section (b), it is expected to obtain between 10 to 20 phage clones that exhibit high affinity binding, to each of the target antibody molecules. Some of these phage will be identical to each other. Ultimately, the aim is to generate approximately five distinct peptide sequences that bind to the antibody. Each of these clones are amplified and sequenced so that peptides can be generated using solid phase chemical synthesis.

(c) Phage clone amplification, sequence identification and peptide production

Large quantities of each of the selected phage are grown and nucleic acid extracted for sequence analysis. Based on the nucleotide sequence coding for the inserted ten-mer peptide, corresponding peptides are synthesized. Numerous commercial vendors provide peptide synthesis capabilities. The binding affinity of each of the selected peptide sequences for their antibodies is determined using Scatchard analysis. The percentage of antibodies binding to the peptide can be ascertained using fluorescence polarization analysis, provided that the peptide is fluorescently labeled. The specificity of binding for each peptide antigen mimic is tested using irrelevant, isotype-matched antibodies.

At the end of the above process, peptide antigens are identified that mimic the binding, characteristics of the monoclonal antibody to the real antigen.

Example 2: Peptide (antigen mimic) - Matrix Optimization

(a) Overview

Once an appropriate peptide sequence is identified, the next step is to determine the linear range of the peptide concentration on the control strips. In other words, how much peptide should be placed onto the nylon strip so as to be most sensitive to early reagent failure?

Carboxy-derivatized nylon or PVDF matrices allow high capacity covalent immobilization of proteins to the surface of the matrix while retaining biological activity. High capacity nitrocellulose membranes also serve to immobilize proteins or peptides in a manner adaptable to this invention. Processing steps include the covalent or non-covalent binding of protein to the membrane and a blocking step to quench the remaining covalent binding capacity, as described herein. Standardization is more readily achieved with proteins that are covalently, rather than non-covalently, adsorbed to matrices. Other desirable characteristics of nylon or PVDF membranes include moderate cost, resistance to microbial attack, and mechanical strength.

(b) Maximizing Sensitivity To Early Reagent Failure.

Described herein is a preferred method for determining the optimal concentration of peptide on the quality control strip. Based on the manufacturers' specifications (Millipore Immobilon-AV Affinity Membrane or Pall Biodyne D membrane), the coupling capacity far exceeds the likely desired peptide concentration. In order to serve as a useful absolute quantitative standard, it is important to precisely control the amount of peptide that is bound to the membrane.

Different laboratories use slightly different concentrations of primary antibody, depending upon the sensitivity of their detection systems. If the purpose of the control strip is to monitor assay sensitivity, then this invention will be configured to bracket a range of peptide concentrations. A series of two to five-fold dilutions for spots of peptide on a single control strip is ideal. The control strip will include a spot with an irrelevant peptide. If the control strips are configured with a series of peptides at the

correct concentrations, then they will be highly sensitive to a decrement in antibody activity. To achieve maximal sensitivity, it is important to identify the dynamic (linear) range for the IHC assay for each peptide/primary antibody combination.

For each peptide/primary antibody combination, dynamic ranges are determined as follows. A series of spots are placed onto a derivatized matrix. Each spot represents a two to five fold dilution of peptide, so that the peptide concentration varies over a broad range. The spots are then detected using primary antibody concentrations already optimized for tissue IHC staining. Typically, primary antibodies in IHC assays are used at a 1-10 microgram/ml concentration. The intensity of the spots can be visually estimated on a 1-4+ scale. Alternatively the spots can be scanned using a flat bed scanner and quantified by densitometry using ImageQuaNT software (Molecular Dynamics Inc.). The staining intensity, or "dot density," is quantified on a numeric scale (arbitrary units).

Figure 4 demonstrates results using the ER peptide mimic (as described herein) and the 1D5 anti-human estrogen receptor antibody. The 1D5 antibody (purchased from DAKO Corporation, Carpinteria, CA) was diluted 1:50 in phosphate buffered saline and incubated for 30 minutes at 37°C. The binding of the 1D5 antibody to the ER peptide mimic was detected using a standard immunohistochemical detection kit. The kit comprises a secondary antibody (anti-mouse IgG-biotin conjugate) and a peroxidase-streptavidin conjugate. This type of detection kit is widely understood to those skilled in the art.

The ideal mean peptide concentration for the center spot on the control strip is that concentration near the juncture of the linear and plateau portions of the graph. From Figure 4, that point is the 4 micromolar peptide concentration. In this particular assay setting, the signal intensity beyond a peptide concentration of approximately 6 μM seems to plateau off, while the lowest threshold of detection appears to be approximately 2 μM. It is at this peptide concentration that the control strips of the present invention will be

most able to detect early reagent failure. If a reagent begins to experience diminished activity, the colorimetric intensity (y axis) will correspondingly diminish (linear portion of curve). On the other hand, operating on the plateau portion of the curve will cause a lack of colorimeric decrement if mild reagent degradation occurs.

Example 3. Validation of Synthetic Control Strips

Each peptide mimic or antigen comprises a somewhat unique and independent product. Therefore, each needs to be independently validated. This section describes the process of validating the synthetic control strips to verify that (a) each synthetic control strip has a high signal to noise ratio, (b) the synthetic control strip is antibody-specific, and (c) each synthetic control strip is capable of detecting early reagent failure.

(a) Measurement of the signal to noise ratio

The synthetic control strip contains two distinct areas: (1) the reagent surface that is devoid of bound peptide, and (2) the test area with specific peptide. The test strip containing no peptide should be used as a test for non-specific binding of antibody to the membrane itself. Excessive background to the negative control (surface without peptide) indicates the need to investigate the blocking procedure, buffer composition, and/or the washing procedure during the assay.

In order to conduct the validation, control strips can be generated with three portions, or regions, on the strip: (1) a portion that contains the relevant specific peptide, (2) a portion that contains an irrelevant peptide, and (3) a portion that does not contain any peptide. The latter two are both negative background controls. Each of the three regions are blocked and washed in an identical fashion. Namely, the strips are blocked in 4% blotto or 10 mg/ml casein) in PBS and washed with PBS with 0.05% Brij 35. The strips are then stained using a conventional immunostaining procedure.

At the end of the immunostaining procedure (but before the addition of the

colorimetric substrate), the three sections of the control strip are separated from each other with a razor blade and placed into microtitre wells. A soluble peroxidase substrate is added to each well (o-phenylenedihydrochloride) with 0.3% H_2O_2. After the color develops, the strips are removed from the wells with a forceps. Color development is measured spectrophotometrically with a microtitre plate spectrophotometer. A signal to noise ratio is then calculated. The first portion of the strip (relevant peptide) represents the signal. The other two portions are both negative controls. A S/N ratio greater than 10 should be obtained for acceptable performance.

(b) Testing for non-specific binding of peptides to other antibodies.

Before settling upon a specific candidate peptide for use as an antigen mimic, it is important to verify that it binds only to the desired antibody. Therefore, it is desirable to test the peptide against an array of 5-10 different primary antibodies. The experimental method is nearly identical to that described above, for determining the signal to noise ratio. Namely, replicate spots of the peptide sequence are coupled onto a matrix (by methods previously described). The spots are separated from each other and placed into microtiter wells, as previously described. Different primary antibodies, each at the appropriate working concentration (usually 1-10 micrograms/ml), are added to the wells. In this manner, the same peptide target is tested against a variety of different primary antibodies. After incubation and removal of the primary antibodies, the remainder of the detection procedure is carried out. An antigen-specific mimic causes a colormetric reaction to occur following incubation with the specific antibody but not by other non-specific primary antibodies.

Table 2 shows representative data obtained during testing of the ER peptide mimic. The peptide was detected using the 1D5 anti-ER antibody (as previously described). In addition, the indicated other primary antibodies were also tested for immunoreactivity to the ER peptide mimic. The color intensity was scored on a semi-

quantitative 1-4 scale, 4 representing intense staining.

Table 2. Immunoreactivity of monoclonal antibodies to ER peptide mimic

Antibody	Staining intensity (1-4+)
1D5 (estrogen receptor)	4
PR636 (progesterone receptor)	0
PD7/26 + 2B11 (CD45)	0
AE1/AE3 (cytokeratin)	0
Normal mouse IgG	0
MIB-1 (Ki-67 antigen)	0

(c) Validating sensitivity of the control strips to detecting early reagent failure.

For optimal performance, the range of concentrations of antigen or peptide mimic (coupled onto the matrix) should be such that it will be most useful in detecting early decrements in reagent performance. This is in contradistinction to standard tissue controls in that the amount of an analyte in a tissue control can not be varied at will. The control strip is most sensitive to detecting early reagent failure when the amount of antigen or antigen mimic on the strip brackets the threshold of detection.

To test the control strips, reagent failure is simulated in a typical immunohistochemical staining procedure. This is accomplished by serially diluting the primary antibody. The limit of sensitivity is measured by visually noting the lowest peptide concentration that will yield a 1+ colorimetric signal. This type of visual quantitation is similar to that used for hemagglutination reactions. The intensity of the reaction (1-4+) is graphed against the concentration of the primary antibody. The goal is to identify the smallest increment in primary antibody dilution that can be detected using the control strips. The sensitivity of the controls strips is

quantified as the smallest increment (change in concentration) in primary antibody dilution that can be reliably detected. (See, for example, Table 3).

TABLE 3

Primary antibody concentration	Colorimetric intensity
40 mcg/ml	4+
20 mcg/ml	4+
10 mcg /ml	4+
5 mcg /ml	3+
2.5 mcg /ml	2+
1.0 mcg /ml	1+
0.6 mcg /ml	0 (negative result)

In this example, the control strip is able to detect a two-fold dilution of primary antibody between 1 and 10 mcg/ml of primary antibody.

The signal intensity on the control strips can also be monitored quantitatively using an image analysis software program, as described for Figure 4. Such a quantitative analysis was performed and the data are shown in Figure 5. Figure 5 summarizes the data from a representative experiment of reagent failure, testing the primary and secondary antibody reagents. Figure 5A illustrates the dot density (y axis) on a series of spots (x axis) containing varying concentrations of ER peptide (to be described later). The control strips are stained with three different dilutions of primary antibodies in Figure 5A. Figure 5B illustrates the signal intensity using three different dilutions of the secondary antibody. In each case, decreasing the antibody concentration (increasing dilution) results in a diminished signal, providing a visual cue that the antibody reagent is less potent. Moreover, the endpoint of detection is also sometimes affected. For example, if the cutoff for positivity (a 1+ visual determination) is a 20,000 dot signal density, then the endpoint of detection in Figure 5B, 1:300 dilution is 1 micromolar

peptide concentration. Using the same standard, the 1:1200 dilution of antibody falls below this point at the 2 micromolar concentration. The 1:4800 concentration never attains even this level of signal intensity (dot density). According to this method, early reagent failure can be monitored by the downward deviation of the spots' color intensity.

Example 4: Identification of Peptide Mimic Of Estrogen Receptor (ER)

Monoclonal antibody clone 1D5 is a widely used antibody that is used for identification of ER in tissue sections by immunohistochemistry. Antibody 1D5 binding peptides (ER epitope mimics) were obtained by screening the cyclic peptide library that is displayed by M13 filamentous phage. The phage library was purchased from Dyax Corp., Cambridge, MA. Figure 6 shows the peptides (SEQ ID NOS: 2-29) and consensus sequences (SEQ ID NOS: 32-42; the underlined amino acids of SEQ ID NOS: 2-29 shown in Figure 6 are referred to herein as SEQ ID NOS: 32-42, respectively) that specifically binds to ER monoclonal antibody 1D5. These ER peptides mimic the three-dimensional structure (conformational epitope) of ER that is recognized by the 1D5 monoclonal antibody. However, other peptides e.g., about 10-20 amino acids long, that comprise (contain) these consensus sequences, SEQ ID NOS: 32-42, be used in the described assays.

Appropriate alterations of the peptides described herein, which retain the peptides's functionality will be clear to a person of ordinary skill in the art. Such alterations include, for example, conservative substitution of amino acid residues, *e.g.*, replacement of a hydrophobic amino acid with a different hydrophobic amino acid, *e.g.*, glycine, valine, leucine or isoleucine. Similarity, negatively charged residues may be replaced, e.g., aspartic acid for glutamic acid, or vice versa. In the case of positively charged residues, arginine may be replaced with leucine, or vice versa. Such alterations do not change the biological activity of the peptide, e.g., the ability of a peptide to bind specific antibody, or the usefulness of the peptide as a quality control moiety in the

assays described herein.

The synthetic ER peptide sequences that were deduced from the selected peptide phage library were further labeled with fluorescein tag for quantitative and qualitative evaluative purposes.

Screening for a peptide mimic for anti-ER antibody.

Two cyclic libraries (TN6 and TN10, purchased from Dyax Corp.) were used to screen for phage that bound specifically to the 1D5 mouse monoclonal antibody (ER-specific antibody). The screening procedure is the same as one described in earlier sections. After two rounds of positive selection, individual phage clones were amplified and used in a phage ELISA. The DNA of representative phage clones (that had the highest ELISA O.D. readings i.e., ~80-90% of the clones in our screen) were sequenced. As shown in Figure 6, clones from each library had a consensus sequence.

Three synthetic peptides (designated as ER peptide 3, ER peptide 4, and ER peptide 6), that are representative of above consensus phage sequence, were synthesized (by SynPep Labs, Dublin, CA and Bachem Labs, King of Prussia, PA) and tested for specificity/affinity to 1D5 monoclonal antibody. Peptides 3 and 6 are shown in Table 4. In a peptide ELISA, ER peptide 3 the highest signal. In a fluorescence polarization assay, ER peptide 3 displayed a Kd of $\sim 10^{-9}$ respectively.

Table 4. Synthetic ER Peptide Mimics

Peptide #3 Ac-DFQCPYVECVVNAPGGK(FITC)GK-$CONH_2$ (SEQ ID NO:30)

Peptide #6 Ac-HSHCQAPYLSMACLPPAGK(FITC)GK-$CONH_2$ (SEQ ID NO:31)

Notes:

(1) The "(FITC)" group represents a fluorescein group attached to an epsilon side chain of lysine.

The carboxy termini of both peptides are comprised of a lysine (K) whereby the carboxy terminus is modified to an amide group.

1. The amino termini of both peptides is acetylated.
2. The consensus regions of both peptides is underlined.

Example 5: Attachment of Peptide Antigen Quality Control Moieties Directly to Glass

In an alternative embodiment of the present invention, antigen is immobilized directly onto the surface of a microscope slide. This is accomplished by placing a small spot of soluble antigen onto one or more spatially discrete regions of the glass surface. In order for the antigen to bind to the glass, the glass is chemically derivatized ("activated") so as to be capable of binding to macromolecules. The front surface of the glass slide (typically the side with a frosted-end on one side of the microscope slide) is referred to herein as the reagent surface. This is the same surface to which a matrix with an adhesive backing would have been attached in the previous embodiment. The reagent surface has one, or more, quality control moieties immobilized to it. The biological sample (e.g., tissue section) to be tested is also mounted to the reagent surface of the slide, preferably adjacent to the control moieties. By being placed adjacent to each other, the tissue section and the quality control moieties are treated with the same reagents, at the same temperature and for the same period of time.

The clinical uses of this alternative embodiment (quality control moieties attached directly to glass slides) are similar to those already described for the previous embodiment (quality control moieties attached to a matrix with an adhesive backing). These uses include detection of early reagent failure, verification of proper assay performance, determination of assay sensitivity, and quantitation of an analyte in a tissue section or cell sample.

There are advantages of attaching the quality control moieties directly to glass slide (second embodiment) instead of a matrix with an adhesive backing (first

embodiment). First, it is possible that immersion of the slide into xylene or alcohol will cause damage to the adhesive backing. This can occur because many adhesives are at least partially soluble in xylene or alcohol. Xylene and alcohol is used for removing paraffin wax from tissue sections and for dehydration before coverslipping. Placing the quality control moieties directly onto the glass circumvents this problem. Second, many IHC procedures require a pre-treatment step, often termed "antigen-retrieval" or "epitope retrieval", that involves immersing the microscope slide in a boiling water temperature aqueous buffer. This boiling can also potentially damage the matrix and adhesive. This problem is also circumvented by attaching the quality control moieties directly to glass, since glass is impervious to the effects of high temperature. Third, the matrix and adhesive backing typically have a small but defined thickness. This thickness causes the reagent surface of the membrane to be at a slightly higher elevation than the glass slide. In order for the reagents to contact the surface of the matrix, there must be a sufficient amount of reagent so that the fluid layer height is greater than the thickness of the matrix and adhesive backing. Many of the assays are performed using only a few drops of reagent. Such limiting volumes of reagent can sometimes be insufficient to contact the matrix surface. Attaching the quality control moieties directly to the glass overcomes this problem as well, since no physical height barrier separates the tissue section and the quality control moieties.

The chemistry of covalently coupling macromolecules to a glass surface.

It is desirable to create a covalent linkage between the silica (glass) surface and the macromolecule serving as a quality control reagent. A covalent linkage is likely to be more stable over a long period of time. Moreover, it is more likely to withstand treatment with solvents and elevated temperatures. Covalent coupling to glass is often accomplished by use of silanes. The silanes of interest are trialkoxy(triiminoxy) silanes and dialkoxy(diiminoxy) silanes. The iminoxy silanes are also called oximinosilanes.

The rest of the valency of silicon is substituted with functionalized alkyl or aryl groups. The dialkoxy (diiminoxy) or the trialkoxy (triiminoxy) groups of the silane are replaced with the Si-OH groups of the glass surface, resulting in the anchoring of the new functionalized silanes. Functionalized trialkoxy (triiminoxy) silanes are preferred since they can crosslink using their three arms and adhere to the glass surface. In order to attach macromolecules, we place a reactive group on the free end of these functionalized silanes. The glass surface before any chemical reaction is represented in Figure 7. Free hydroxyl groups are attached to silicon atoms that form the glass surface.

The first step in chemically activating glass slides is to clean them. Slides can be cleaned by one of several alternative methods: incubation in acid (e.g., 2N HCl or 2N H_2SO_4), or incubation in a base (e.g., 10% NaOH or 10% KOH), or incubation in a cleaning solution (10% Aquet in water, VWR Scientific) for 10-15 minutes. Glass slides were further rinsed by dipping ten times in distilled water and then dried for 2-5 minutes in a 60°C oven. Slides treated in this fashion are referred to herein as "cleaned" slides.

In a one-step method of activating the glass surface (for attachment to macromolecules), an isocyanate end-capped trialkyl siloxane (e.g., 3-isocyanato propyl triethoxysilane) is incubated with the glass slide. This molecule has both a siloxane attachment site (for attachment to glass) and an isocyanate group (for attachment to a macromolecule). A chemical representation of the isocyanate end-capped trialkoxylsilane-derivatized glass surface is shown in Figure 8. The free isocyanate groups are highly reactive and serve to immobilize macromolecules to the glass surface.

To perform the one step activation of glass slides, 3-isocyanato propyl triethoxysilane is dissolved in acetone, toluene, or ethanol at a concentration of 2-10%. The cleaned slides are then immersed in the 3-Isocyanato propyl triethoxysilane solution for 30-60 minutes with occasional stirring. The slides are incubated at 37°C - 45°C which functions well to activate the glass. The coated glass slides are then dipped three times in acetone and dried in a 60°C oven for 5-10 minutes. A potential drawback to this

method is that the free isocyanate group can compete with the alkoxy group of the silane for attachment to the glass. If this occurs, the isocyanate group is not available for attachment to the macromolecule, since it is bound to the glass surface. This can ultimately lead to poor incorporation of the macromolecule onto the glass surface. Therefore, a two step sequence for incorporating the isocyanate groups on to glass surface is also described herein.

In the two step method of chemically activating glass slides, the glass surface is first derivatized to form an amino triethoxysilane, forming an amino end-capped glass surface. This amino end-capped silane-derivatized glass surface is shown in Figure 9. Cleaned and dried slides are coated with a commercially available silane solution (e.g.,3-aminopropyltriethoxysilane or N'-(2-aminoethyl)-3-aminopropyltriethoxysilane or cyanosilane etc.,) at a 2-10% concentration (in any of the following solvents: acetone, a 95:5 ratio of acetone-ethanol, toluene, di or tri-chloromethane, ethanol, or methanol). The reaction of the glass slides with the silane solution occurs at 37-45^{0}C for 30 minutes, with occasional mixing. Silane-coated glass slides are then dipped three times in acetone and dried in a 60°C oven for 5-10 minutes.

The amino groups on the glass slide are then subsequently converted to reactive isocyanate groups by one of two methods. In the first method, the amino group can be reacted with a molecule containing -diisocyanate. One cyanate group (of the diisocyanate) reacts with the amino end-cap to form a urethane bond. The other isocyanate group is available for reacting with the quality control macromolecule. The following diisocyanates will work for this purpose: phenylene diisocyanate, 1,4-diisocyanato butane, 4,4'-diisocyanato dicyclohexyl methane (See e.g., Figure 10). For example, incubation with a 0.5-1% solution of 1,4 phenyl diisocyanate, dissolved in acetone or toluene, for 30 minutes at 37-42°C will react with the amino groups on the glass. One possible problem that can arise with a homobifunctional cross-linker (such as a diisocyanate) is that both reactive (isocyanate) groups react with the surface. Cross-

linking surface-bound amino groups (by the two arms of the diisocyanate) is avoided by adding the diisocyanate in molar excess. The resulting mono isocyanate end-capped glass surface is useful due to its reactivity towards coating of biological molecules especially proteins (or peptides), nucleic acids (DNA) and polysaccharides. Two modes of chemical mechanism of attachment, via the isocyanate group, are shown in Figures 11 and 12.

Instead of using a diisocyanate, the amino end-capped glass surface can also be reacted with monomeric poly-isocyanates such as triisocyanatobenzene or tetraisocyanatobenzene or their homologues such as tetraisocyanato toluene. In this way, an even greater number of active isocyanate groups are incorporated on to the glass surface.

The previously described methods converted the amino end-capped silane to a cyanate by using either a diisocyanate or a poly-isocyanate. In an alternative method, the amino end-capped silanes on glass can be converted to an isocyanate group by treating the amino end-capped glass surface with phosgene equivalents, like 1,1'-carbonyl diimidazole or oxime carbonates (e.g., methyl ethyl ketone oxime carbonate). This latter alternative (with phosgene equivalents) is preferred over the former alternative (adding a diisocyanate) because of its higher reactivity under milder conditions and avoidance of the possibility of crosslinking the surface amino groups. In addition, the materials are safer to handle, an advantage in manufacturing.

The isocyanate groups on the glass will readily react with an amine (e.g., found in short peptides, proteins, and nucleic acids), carboxyl group (e.g., found in short peptides and proteins) or hydroxyl group (e.g., found in carbohydrates and sugars). Since the isocyanate groups on activated slides are highly reactive, the treated glass slides should be stored in a anhyhydrous environment. Alternatively, the glass incorporated isocyanate groups can be blocked with an oxime such as methyl ethyl ketoneoxime (MEKO). The labile MEKO-blocked isocyanates lose MEKO to reliberate

activated isocyanates in the presence of amine-bearing peptides to form stable urethanes at room temperature. Alternatively, MEKO can be removed at elevated temperatures (80-120°C), liberating the glass-attached free isocyanate groups.

These glass activation chemistries also provide for the possibility of creating "blocked" isocyanate groups on the glass slides. This was previously briefly mentioned with respect to methyl ethyl ketoneoxime (MEKO). If the isocyanate groups are not blocked, then the isocyanate group is so reactive that it can potentially react with water. Over time, even the humidity in the air can react, degrading the reactivity of the isocyanate groups. To maximize the stability of slides with unblocked reactive isocyanate groups, it will be ideal to store such slides in a hygroscopic environment. The advantage of the blocked reactive group is that it will provide for a longer shelf life. The blocked isocyanate groups that we will describe are unreactive with water at room temperature but will react with groups such as amines, as would be found in proteins, carbohydrates, or nucleic acids.

Three blocking methods are described. In all three methods, the glass is first reacted with 3-aminopropyltriethoxysilane, as previously described. The triethoxysilane functionality reacts with the silicon atoms of the glass, leaving a free primary amine group covalently attached to the glass. In the first method, the amine-derivatized glass is then reacted with 1,1-carbonyldiimidazole (10%) at 40°C for 30 minutes. In the upper half of Figure 13, the derivatized glass surface is shown, bearing a free amino group. The bottom half of Figure 13 shows the structure of the product after the reaction with the carbonyldiimidazole. The 1,1-carbonyldiimidazole reacts with the amino group on the glass, forming a mildly stable intermediate shown in Figure 13. The imidazole-protected isocyanate is mildly stable at room temperature. Namely, the attached imidazole group will not leave in the presence of water (unless the temperature is raised above 60°C). However, the protecting group will leave in the presence of an amine. Thus, the glass surface is reactive towards biologically-relevant macromolecules but

does not have the problem of instability to water, such as humidity. After reacting with an amine, the final product appears as in Figure 11 or 12.

A second method forming a protected isocyanate group is to modify the glass-bound amine group with MEKO Carbonate or oxime carbonate. The upper half of Figure 14 shows a glass-bound amine (on the end of aminopropylsilane) reacting with MEKO Carbonate. The lower half of Figure 14 shows the oxime-blocked isocyanate group. This oxime-blocked isocyanate group is reactive towards amines, as described previously for the imidazole-blocked isocyanate, but towards water at room temperature. Upon contact with an amine group, the oxime group is displaced to form a urethane linkage. In this manner, the activated surface is unreactive towards water but reactive with a biologically relevant macromolecule, such as a protein or nucleic acid. After reacting with an amine, the final product appears as in Figure 11 or 12.

Coupling the macromolecule that serves as the quality control reagent is accomplished by incubating a small aliquot (approximately 1 microliter) of the macromolecule dissolved in an appropriate coupling buffer (e.g., 0.1 M potassium phosphate buffer, pH 7.5) on the activated glass surface for 5-30 minutes at room temperature or 37°C. Typically, small spots containing the macromolecule are placed onto the surface of the glass. The use of multiple discrete spots positioned on the glass slide is similar to that already described for conjugation to matrices such as membranes. The spotting of the antigen could be done either by manually, with a pipette, or with a commercially available liquid dispenser.

Alternatively (instead of dots), the various peptide/protein controls can be marked as letters indicating the test antigen. For example, the progesterone receptor control spots can be calligraphed as "PR" in place of dots.

After the coupling procedure, the remaining reactive sites on the chemically activated glass slide must be "quenched" (or "capped"), blocking any future potential reactivity. Reactive sites could otherwise directly attach to components of the assay,

such as antibodies, causing background staining. It is possible to leave the slide unquenched until after the tissue has been mounted onto the glass. In this manner, the isocyanate groups can bind to amino or carboxyl groups on tissue proteins. This may help anchor the tissue to the glass slide. After the tissue is mounted on the glass, the remaining unreacted isocyanate groups can then be quenched.

The glass surface is quenched with any of a variety of small molecules that have free amino groups. Suitable quenching agents include ethanolamine, ethylenediamine, aminoethanediol, aminopropanesulfonate and dithiothreitol. Alternatively, proteins such as gelatin or casein or amino acids such as glycine, can quench the active glass surface. recommended quenching procedure is to cover the glass slide with 10% v/v monoethanolamine in 1.0 M Sodium Bicarbonate buffer, pH 9.5. The glass slide is incubated with the quenching solution for 5-30 minutes at room temperature or 37°C. The quenching solution is then drained and the glass slide is rinsed with 0.01 M Sodium Phosphate, 0.14 M NaCl, pH 7.4, 0.1% v/v Tween 20 (PBS-Tween).

Equivalents

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.